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6. AUTHOR(S)

Rene J. Herrera, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Florida International
University
11200 SW 8th Street MARC 430
Miami, Florida 33199

8. PERFORMING ORGANIZATION REPORT NUMBER

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14. ABSTRACT The unusual combination of strength and extensibility of the *Nephila clavipes* spider dragline silk greatly surpasses all currently known high-performance synthetic materials. Together with the fact that silk is biocompatible and biodegradable, this natural polymer constitutes a very promising fiber for novel applications including use in bullet-and explosion-proof clothing and highly resistant surgical thread. Yet, massive production of this fiber from natural sources is not feasible so far. Other alternatives like production of the protein that yields same or similar mechanical properties of dragline silk in microorganisms or mammalian cells, in spinning fibers from concentrated protein solutions, have failed or are inefficient. Currently, silk is produced from the cocoon of the silk moth *Bombyx mori*; however, this silk has about one-tenth the strength and flexibility of *Nephila clavipes* silk. The goal of this proposal is to develop a transgenic silk moth able to produce *Nephila clavipes* dragline-like silk. In order to do this, a chimeric gene called Spidrofibroin (SpF) have been constructed. SpF combined the repetitive domains of spider dragline silk with the N- and C- terminal domains of the *Bombyx mori* silk gene, Fibroin-H (Fib-H). Various SpF genes have been cloned under the promoter of the Fib-H gene in pBac vectors and used to generate silk moth embryos. The silk fibers spun in the transgenic silk moth cocoons are being analyzed with respect to expression of SpF and mechanical properties of the resulting fiber. Since the SpF variants will have all the necessary elements for expression, transport and assembly into the silk fiber of *Bombyx mori* as Fib-H does, we believe that SpF will compete with Fib-H for its assembly into the silk fiber.

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Final Performance Report

I. Objectives

In an attempt to find an economical method to produce large amounts of stronger, more flexible silk for commercial use, our work has taken advantage of the superb qualities of spider silk, particularly that of *Nephila clavipes*, the ability to produce transgenic insects and the biosynthetic capacity of the domesticated silkworm, *Bombyx mori*. The elasticity and strength of spider silk make it ideal for incorporation into fabrics used to produce parachutes, explosion/bullet-proof vests, and surgical sutures. However, given the aggressive and territorial nature of spiders and their limited biosynthetic capacity to produce silk, raising spiders to manufacture silk is not commercially practical. On the other hand, *Bombyx mori* is already used globally on an enormous scale for silk production. *Bombyx mori* rearing requires small amounts of space as well as minimal personnel effort and attention. This research focused on developing a strain of transgenic *Bombyx mori* that contains a chimeric silk gene comprised of the genetic regulatory elements from *Bombyx mori* fibroin and segments of the *Nephila clavipes* spider silk gene that confer enhanced strength and flexibility inserted between the targeting and assembly domains of *Bombyx mori* fibroin for the production of a superior commercial silk.

II. Status of Effort

This final performance report outlines the advances that have been made during the entire funding period. These experiments included 1) the construction of three SpF chimeric transgenes, each with a variable number of repetitive sequences from the *Nephila clavipes* Major Ampullate Gland (MAG) silk gene that were inserted into a densovirus-derived (JcDNV) somatic transformation vector and *piggyBac* transposon derived (pB) germline vectors; 2) utilizing these vectors to create transgenic insects including the silk producing Lepidopteran *Plodia interpunctella*, the fruit fly *Drosophila melanogaster* and the silkworm, *Bombyx mori* to assess the functionality of the chimeric silk transgenes; and 3) the preliminary analysis of proteins produced in the silk glands of the transgenic moths by SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE).

III. Accomplishments

Aim 1: Construction of the SpF transgenes.

A. Synthesis of the repetitive domains of the MAG silk gene: The repetitive region of the MAG gene was fully synthesized by blunt-end ligation of machine generated repetitive motifs. This was accomplished through repeated cycles of ligation, transformation and sequencing, which produced identical repeats of the MAG consensus repetitive motif. Three unique transgenes were constructed containing different numbers of repetitive motifs in the *Nephila clavipes* region of the SpF. These three transgenes possess 542, 567 and 945bp, respectively from the *Nephila clavipes* repetitive domain.

By using synthetic repetitive motifs of the MAG gene, we avoided the natural variability in length and sequence of the repetitive domains. This variability may have some influence on the structure of the fiber and its resulting mechanical parameters. Another advantage of synthesizing the repetitive motifs is that we were able to take advantage of the codon usage of *Bombyx mori* (Zhou et al., 2000). In this way, expression of the *Nephila clavipes* MAG region is maximized in *Bombyx mori*.

B. PCR-amplification of the 5' and 3' flanking regions, and N-and C-terminal domains from *Bombyx mori* fibroin gene. All modules originating from *Bombyx mori* Fib-H were PCR-amplified from genomic DNA using specific primers. The 5'-flanking region containing the *H-fib* promoter (-240 to 0 of the *H-fib*) was PCR-amplified together with the nucleotides for the first 14 amino acids of Fib-H which includes the N-terminal domain. The second fragment of the N-terminal domain is 137 amino acids long (411 bp) and is located from bp position +1038 to +1449. The C-terminal domain is 50 amino acids long (150 bp) and is located at the 3' end of the *H-fib* gene +16,740 to +16,790. This fragment was amplified together with the 3' flanking sequence of the *H-fib*. Following purification of PCR products, the gene modules were cloned in the pCR-II[®] TOPO vector and white colonies from blue/white screening were selected for sequencing. These gene modules were assembled by blunt-end and/or sticky-end ligations, faithful to the sequence and proper reading frame. These constructs include different numbers of the *Nephila clavipes* repetitive domain to generate the three transgenes. SpF variants were removed from the TOPO vector using the *EcoRI* and *HindIII* restriction enzymes and ligated into *BamHI*-linearized *piggyBac* vectors containing an hr5IE1DsRed or 3xP3EGFP reporter gene for scoring transgenesis.

Aim 2: Somatic transformation of SpF into insect cells using JcDNV.

A. JcDNV Constructs. The JcDNV somatic transformation vectors are based on the recombination of plasmids that contain viral sequences with host cell chromosomes (Royer et al. 2001; Bossin et al., 2003; Bossin et al. Submitted). Three different SpF constructs with varying amounts of *Nephila clavipes* repetitive elements from the MAG gene were PCR-amplified from the pGEM plasmids containing the constructions and inserted into the pJDsRedMCSH vector (Bossin et al., 2007) and microinjected into the syncytial embryos of three insect species.

Three trips were made to the laboratory of our collaborator, Dr. Paul Shirk at USDA ARS CMAVE, Gainesville, FL, to perform genetic transformations of insect eggs by microinjection utilizing both somatic and germline transformation vectors. Initial experiments were conducted in the Indian meal moth, *Plodia interpunctella* and the fruitfly, *Drosophila melanogaster*. These two insects provided readily available and useful models with which Dr. Shirk has extensive experience. Somatic transformation of a subject larva was scored by visualization of DsRed expression from the integrated transforming construct. Expression of the DsRed were observed in late embryos and larvae originating from microinjected *Plodia interpunctella* embryos. Eggs that fluoresced in high amount were screened using a fluorescent dissecting microscope and chosen to continue larval development.

The pJDsRed[BmNcS] somatic transformation vector was microinjected into syncytial embryos of both *P. interpunctella* and *D. melanogaster*. In the previous *Plodia* experiments, 1,535 embryos were microinjected and 486 (31%) showed DsRed fluorescence. There was only a 6% (91 larvae total) hatch rate (compared with 17% for control injections) in this experiment. Of the surviving larvae, 75% (69 total) were transformed and maintained through larval development. A normal pattern of P9 driven DsRed expression was observed in these larvae. For *Drosophila*, 248 embryos were microinjected and 74 (30%) showed DsRed fluorescence. There was a 25% (63 total) hatch rate with 17% of the larvae somatically transformed. When the *Plodia* larvae were spinning silk for cocoons in the last instar, the salivary glands were collected and the secretions were analyzed by SDS-PAGE.

Aim 3: Analysis of SpF protein expression.

In order to confirm successful transformation of *Plodia interpunctella*, posterior silk glands were dissected and the liquefied silk from its lumen was aspirated into a micropipettor. This procedure was repeated in order to accumulate several microliters of purified, liquefied silk. Due to the highly specialized function of the silk gland which produces exclusively silk, this method allowed us to collect sufficient quantities of liquefied silk for protein expression profiling. Subsequently, SDS-PAGE analysis was performed using these extracts from the lumen of the posterior silk gland. Lumen extract from recombinant larvae were compared against extracts from non-transformed larvae. Lane 2 containing extract from a transformed larvae shows a distinct difference in protein expression with a band appearing at about 83 kDa as compared to the wild type control with bands at 85kDa, 81kDa and 78kDa. Furthermore, extracts from recombinant larvae in lanes 6 and 9 exhibited a unique band at 71kDa not observed in the wild type control in lane 3. This is indicative that not all transformed *Plodia interpunctella* individuals exhibit the same protein expression from the lumen of the posterior silk gland. Sequencing of each of these bands, from both transformed and non-transformed larvae is currently underway.

Aim 4: Stable germline integration of SpF variants into *Bombyx mori* Nistari strain eggs.

Our experimental methods were applied and tested first in *Plodia interpunctella* and *D. melanogaster* with promising results. Subsequently, we performed germline transformation of *Bombyx mori* eggs using a *piggyBac* transposon gene vector. The *piggyBac* vector contained a hr5IE1DsRed expression cassette as a marker for transformation and the phsp-pBacwc as helper (Bossin et al., 2007). The hr5IE1 promoter resulted in high levels of expression in late embryos and early larvae. During a third visit to Dr. Shirk's laboratory, 800 *Bombyx mori* Nistari strain eggs were injected with the three *piggyBac*-SpF constructs. The injection sites were sealed with super glue, and the developing embryos were observed for somatic transformation. From this experiment, 332 larvae hatched (45%) and 95 adults were mated. Unfortunately no germline transformants were recovered from this experiment. Because the transformation efficiency for this vector in the *Bombyx* Nistari strain is 1% (Tamura et al., 2000; Thomas et al., 2003), it was not unexpected that transformants were not recovered. This experiment did provide a reasonable run-through to assess the transformation and rearing protocols. The DsRed fluorescent proteins were detected using a Leica MZ FLIII fluorescent stereozoom microscope using a

GFP/DsRed dual filter set (Excitation 470/30 nm and 565/20 nm; Barrier 510 nm and 580; Emitter 535/45 nm and 610/60 nm). Digital photographs of the insects were made using a Leica DC 500 CCD camera operated with Leica IM50 software.

Our most recent set of experiments were conducted with a *piggyBac* that contained the 3xP3EGFP expression cassette (Horn & Wimmer, 2001) to take advantage of the expression of GFP in embryonic and larval ocelli (eyes) (Thomas et al., 2003). In these latest efforts at transformation using the GFP reporter gene, a total of 2,060 fertilized *Bombyx mori* eggs were injected. The development of the microinjected embryos corresponded with that of the control embryos in spite of the wounding from the microinjection. The ocelli (eyes) were seen clearly in the head capsule of the 10 day old embryos. Somatic transformed cells in embryos from days 4, 6 and 10 were observed. Unfortunately none of these embryos hatched.

The positive preliminary results from the experiments in collaboration with Dr. Shirk's laboratory at the USDA ARS in Gainesville Florida led to our acquisition of \$26,000 in matching funds from the USDA to procure a fluorescent dissection microscope equipped with high-sensitivity DsRed and GFP filters in order to screen broods of *Bombyx mori* eggs and larvae resulting from future injections.

IV. Personnel Supported

Dr. Jose Luis Coll, Ph.D., Postdoctoral Fellow

V. Publications

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3. Suarez-Martinez, E. B., Montealegre, F., Sierra-Montes, J. M. and Herrera, R. J. (2005). Molecular identification of pathogenic house dust mites using 12S rRNA sequences. *Electrophoresis.* 26: 2927-2934
4. Sierra-Montes, J. M., Pereira-Simone, P., Freund, A., Ruiz, L., Szmulewicz, M. and Herrera, R. J. (2005). The silk moth *Bombyx mori* U1 and U2 snRNA variants are differentially expressed. *Gene.* 352: 127-136.
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6. Somarelli, J. A. and Herrera, R. J. (2007). Evolution of the 12 kDa Fk506-binding protein gene. *Biol. Cell.* 99: 311-321.
7. Somarelli, J. A., Coll, J. L., Martinez, L., Velandia, A. and Herrera, R. J. (2007) Characterization of Immunophilins in the silk moth *Bombyx mori*. *Arch. Insect Biochem. Phys.* 65:195-209.
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9. Somarelli, J. A., Skolnick, J. and Herrera, R. J. (2007). Structure-based classification of 45 FK506-binding proteins. In Press: *Proteins: Structure, Function and Bioinformatics.*
10. Mesa, A., Somarelli, J. A., and Herrera, R. J. (2007). The small nuclear RNA variants of three *Bombyx mori* strains. In Press: *Entomological Research.*
11. Smail, S. S., Mesa, A., Ares, A. L., Rodriguez, A. and Herrera, R. J. (2007). U2 snRNA isoforms differentially associate with high molecular weight spliceosomes in the silk moth *Bombyx mori*. Submitted to *Insect Molecular Biology.*
12. Mesa, A., Somarelli, J. A., and Herrera, R. J. (2007). Spliceosomal Immunophilins. Submitted to *Biol. Cell.*

VI. Interactions/Transitions

1. Spider silk production by silk moths. Plenary Seminar. Air Force Research Conference. January 2005
2. Spider silk production by silk moths. Plenary Seminar. Air Force Research Conference. January 2006
3. Spider silk production by silk moths. Plenary Seminar. Air Force Research Conference. January 2007